Neuron, Vol. 24, 1017–1028, December, 1999, Copyright ©1999 by Cell Press

Identification of a Novel Process Limiting the Rate of Synaptic Vesicle Cycling at Hippocampal Synapses

Charles F. Stevens* and John F. Wesseling†
Howard Hughes Medical Institute and
Molecular Neurobiology Laboratory
The Salk Institute
La Jolla, California 92037

Summary

During intense presynaptic activity, the readily releasable pool (RRP) of synaptic vesicles empties more quickly than it can be refilled, and short-term depression results. Ordinarily, the pool refills within 20 s, but long, high-frequency trains of action potentials often induce a form of short-term depression that persists for a much longer time. Here, we report that replenishment of the RRP is governed by two simple processes: the previously identified mechanism termed refilling, and another process that appears after extensive exocytosis and produces a transient decrease in the capacity of the pool, lasting for several minutes. The data presented here place stringent constraints on the types of kinetic models that can be used to describe synaptic vesicular cycling and are inconsistent with the traditional multipool models of vesicular mobilization.

Introduction

Synaptic transmission occurs when an influx of calcium, produced by an action potential, triggers the exocytosis of neurotransmitter-filled vesicles. Although a synaptic terminal contains many vesicles, it can release only one or a few at once (Katz, 1969; Zucker, 1989; Korn and Faber, 1991; Tong and Jahr, 1994; Stevens and Wang, 1995). These released vesicles are drawn from a physiologically defined readily releasable pool (RRP), which contains a small fraction of the total number in the terminal (Birks and MacIntosh, 1961; Elmqvist and Quastel, 1965; Katz, 1969; Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996; Schikorski and Stevens, 1997). Rapid trains of action potentials cause the synapse to expend the releasable vesicles more rapidly than they are replaced—the replacement process is termed refilling—and so such episodes of activity eventually deplete the pool (Zucker, 1989; Rosenmund and Stevens, 1996). Since the exhausted pool cannot provide vesicles for exocytosis, a form of short-term synaptic depression, termed depletion, results. During periods of rest, the synapse recovers from depression as the pool refills.

After being emptied, the RRP at hippocampal excitatory synapses refills completely in <30 s (Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996; Stevens and Wesseling, 1998). Several investigators have found, however, that presynaptic depression can persist for several minutes after lengthy stimulation at various types of synapses (Birks and MacIntosh, 1961; Elmqvist and Quastel, 1965; Magleby, 1987; Zucker, 1989; Abbott et al., 1997). Some recent reports indicate a component of synaptic depression may not be caused by a reduction in the content of the RRP pool at all, but rather, in some cases, by a reduced capability of action potentials to trigger the release of fusion-competent vesicles (Takahashi et al., 1996; Brenowitz et al., 1998; Bellingham and Walmsley, 1999). Nevertheless, evidence from the classic literature shows that, at least at the neuromuscular junction, some types of depression are associated with a reduction in the readily releasable store of transmitter that lasts for several minutes (Birks and MacIntosh, 1961; Elmqvist and Quastel, 1965).

The longer lasting depression has never been fully characterized, since the recovery of synaptic strength varies widely from situation to situation and appears to have a complex time course (Liley and North, 1953). A recovery time course of synaptic strength that seems complicated might, however, have a simple basis that is obscured by the superposition of several processes. For example, the type of stimulation that results in slowly recovering depression also produces fairly long-lasting forms of potentiated release from the RRP (augmentation and posttetanic potentiation) that can confound the determination of recovery from depression (Stevens and Wesseling, 1999).

In a recent study, Liu and Tsien (1995a, 1995b) reported on a population of vesicles at cultured excitatory hippocampal synaptic terminals that, once released, takes several minutes to recover. These investigators induced exocytosis by depolarizing the terminals for extended periods with superfused, calcium-containing hyperkalemic solutions. They fit their recovery time course to a single exponential with a 40 s time constant, far longer than the 5–10 s characteristic refilling time of the RRP at these same synapses (Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996; Stevens and Wesseling, 1998). The population of vesicles that Liu and Tsien studied is probably equivalent to several RRPs' worth of transmitter, since they report releasing about 90 vesicles per synapse, whereas Stevens and coworkers observed a RRP size averaging around 10 quanta (Geppert et al., 1994; Stevens and Tsujimoto, 1995; Dobrunz and Stevens, 1997; Murthy and Stevens, 1999). Liu and Tsien's work suggests that, like the neuromuscular synapses studied in the classic work, the releasable population of vesicles at hippocampal synapses sometimes recovers more slowly after extensive activity compared to the usual, relatively rapid refilling of the RRP.

Several workers have speculated that the longer recovery times from depression caused by extensive activity might reflect the depletion of "reserve" vesicles upon which the RRP draws when it refills (Birks and MacIntosh, 1961; Elmqvist and Quastel, 1965; Liu and

*To whom correspondence should be addressed (e-mail: cfs@salk.edu).
† Present address: Cellular Biochemistry and Biophysics, Sloan Kettering Institute, New York, New York 10021.

Tsien, 1995a, 1995b). These classical "mass action" models generally suppose the RRP refills at a rate that depends upon the fullness of a secondary store of transmitter. The RRP would thus refill more slowly as the reserve pool was emptied, accounting for the longer persistence of depression after the more extensive stimulations. The classical models of synaptic vesicular mobilization have never been the only option, however. The slowly recovering depression could, alternatively, be due to some other type of activity-dependent modification of the RRP itself (Elmqvist and Quastel, 1965; Zucker, 1989). Until now, the various models of vesicular mobilization have never been compared quantitatively because the depressive effects of extended activity could not be separated from the various forms of enhancement (Stevens and Wesseling, 1999).

Recently, it has become possible to monitor the contents of the RRP in a way that is uncontaminated by calcium-mediated mediated modulation of the physiological release process (Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996; Stevens and Wesseling, 1998, 1999). This technique has allowed us to resolve the processes that limit the rate at which vesicles become available for release. We report here that, in addition to the previously identified refilling of the RRP (Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996; Stevens and Wesseling, 1998), a second bottleneck is present in the vesicular exo/endocytotic cycle. This limit on the rate of synaptic vesicular exocytosis becomes apparent after extensive synaptic use and then dissipates steadily over several minutes during periods of rest. After depletion, the RRP recovers with a time course described by the weighted sum of two exponentials, one with the previously reported (approximately) 10 s time constant of refilling, and the other with a longer 60 s time constant. The relative weighting of these two components depends on the recent history of exocytosis (the weight of the slow component increases with heavier synaptic use), and so the total time for recovery becomes greater with continued synaptic use. These observations place stringent constraints on the types of kinetic models that can be used to describe synaptic vesicular cycling. In particular, we show how these observation are inconsistent with the traditional model.

Results

For these experiments, we induce depression with long trains of action potentials in cultured, isolated hippocampal neurons and then monitor the recovery of the readily releasable population of vesicles with repeated applications of hypertonic solution. Although exocytosis of synaptic vesicles normally occurs in response to calcium influx into the presynaptic terminal, the contents of the pool can also be released in a calcium-independent manner when the terminals are challenged with a suitable hypertonic solution (Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996; Stevens and Wesseling, 1998). Such an osmotic shock does not alter the postsynaptic quantal response size (Bekkers et al., 1990; Stevens and Tsujimoto, 1995; Stevens and Wesseling, 1998), and so the contents of the pool can be measured up to a proportionality constant by simply recording the synaptic currents evoked by hypertonic challenge. With this technique, we are able to follow the time course of recovery of the RRPs of a population of synapses after lengthy trains of action potentials. These experiments involve recording from isolated hippocampal neurons grown in tissue culture on small "islands" (Segal and Furshpan, 1990; Bekkers and Stevens, 1991). Because the dendrites of our isolated neurons only receive synaptic input from their own axons (autapses), we can be sure that all of the synapses whose properties we examine by application of hypertonic solution previously had experienced action potential–evoked release. By challenging patch-clamped, isolated neurons with hypertonic solution, then, we are able to monitor the contents of the RRP at various times after electrical stimulation of the synapses with a method that does not conflate the recovery process and synaptic enhancement produced by the measurement of the RRP (Stevens and Wesseling, 1999).

Long, High-Frequency Stimulation Induces a Slowly Recovering Component of Depression of the RRP

Our main observation is illustrated in Figure 1, where we display the recovery of the RRP after long, high-frequency trains of action potentials. For each experiment, we induced action potentials in the autaptic neurons at 9 Hz with either 150 or 1000 pulses. Both stimulations were easily sufficient to effectively empty the RRP (see Rosenmund and Stevens, 1996). The autapses were then challenged with hypertonic solution two times in succession. The first challenge was applied at various times after the electrical stimulation and provided an estimate for the total amount of recovery versus resting time. The second application was presented 3 min after the first, when the pool had recovered completely, and defined the resting capacity of the RRP. The time course of recovery, then, was estimated by comparing the measured fractional recovery at each time to the length of delay between the end of the electrical stimulation and the beginning of the first hypertonic challenge.

The autapse preparation often exhibits a small amount of continual "rundown" in the size of the RRP, just as there can be a slow decline in the size of the evoked synaptic currents (Stevens and Wesseling, 1998). Had we not taken it into account, this phenomenon of rundown would have resulted in a slight overestimate of the rate of pool recovery because the normal size of the pool was always measured 3 min after the depressed size. To gauge accurately the recovery time course of the RRP, we therefore independently measured the amount of rundown over a 3 min period for these autapses (average 5% per 3 min) and corrected the data accordingly.

Both of the tested stimulus trains (150 and 1000 pulses) caused a decrease in the size of the RRP that lasted for several minutes—much longer than the refilling time course for the RRP (Stevens and Tsujimoto, 1995). The first data point was taken 10 s after the prolonged stimulation, time enough for the previously reported refilling process to have mostly run its course (Stevens and Wesseling, 1998). Nevertheless, the RRP had not recovered to its resting size by that time, an

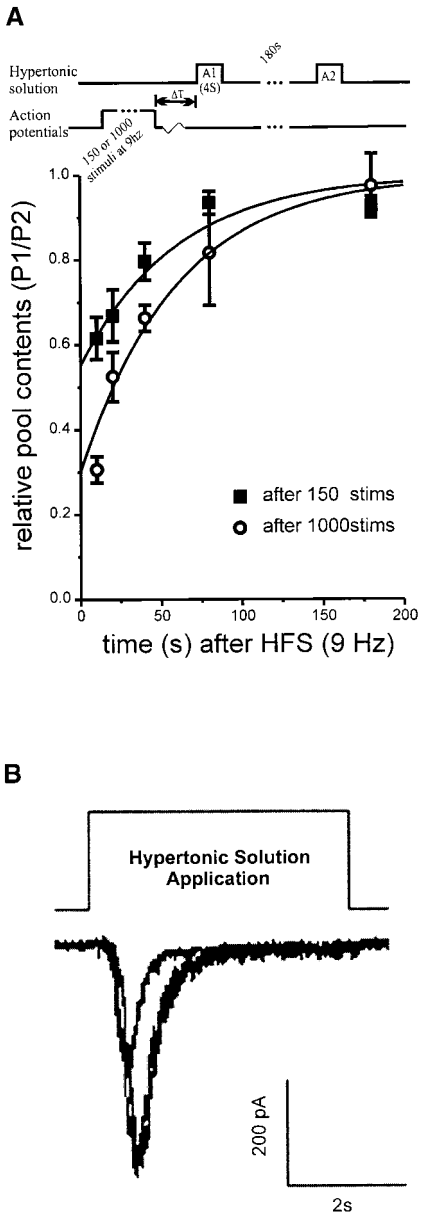

Figure 1. Identification of a Slowly Recovering Component of Depression of the RRP

(A) Depression was induced in autapses with high-frequency stimulations (9 Hz) of either 150 (squares) or 1000 (circles) action potentials. The recovery of the RRP was then monitored with pairs of hypertonic solution applications. The first (A1) was applied at an experimentally varied time after the end of the electrical stimulation, and the second (A2) was applied 3 min later when the pool had recovered completely, as illustrated in the diagram at top. The fractional recovery at each time point was calculated as the pool size estimated at A1 (P1) compared to the size estimated at A2 (P2). Data were corrected for rundown as indicated in the text. The recovery (mean ± SEM, at least six observations [squares] or at least three observations [circles] for each data point) is fitted by a single exponential with a time constant of 60 s in both cases (solid lines). (B) Example electrophysiological data recorded during hypertonic challenges given to monitor the recovery from depression. Overlaid are three traces, the first taken before the induction of 1000 action potentials at 9 Hz, the second (smaller amplitude deflection) taken 20 s after stimulation, and the third taken 3 min later.

observation confirming earlier work (Birks and MacIntosh, 1961; Elmqvist and Quastel, 1965; Magleby, 1987; Zucker, 1989; Liu and Tsien, 1995a, 1995b) that suggested a decrease in the RRP content is at least partially responsible for the persistence of a long-lasting type of short-term synaptic depression. For both stimulus trains, starting 10 s after stimulation, the recovery of the number of releasable quanta can be described by a single exponential with a time constant of 1 min. After 1000 action potentials, the single exponential curve fitted to the data intercepts the ordinate at about 30%, whereas after 150 action potentials it intercepts at about 55%.

Under our conditions, the trains of action potentials did not produce large permanent changes in pool size (see Figure 1B). High-frequency stimulation of cultured hippocampal synapses has been shown to produce very long-lasting changes in synaptic strength under some conditions (Bekkers and Stevens, 1990; Goda and Stevens, 1996, 1998; Ryan et al., 1996), and so we designed these experiments to measure only the transient forms of activity-induced depression. We monitored the recovery after electrical stimulation by comparing the size of the RRP after various delays to the size of the completely recovered pool 3 min later. We did not include comparisons of the size of the pool before and after stimulation in the analysis presented above. In many cases, this information was available, however, because we could maintain stable recording conditions for many trials. A comparison of the RRP size 1 min before electrical stimulation to the pool contents 4 or 5 min later shows an average decrement in pool capacity of only 4% (± 2% SEM, 32 trials) when we stimulated with 150 action potentials, and 10% (± 2%, 12 trials) when we stimulated 1000 times. Although some of this decline might result from the induction of a small amount of long-term depression, most of it probably represents the time-dependent rundown mentioned above.

Recovery Proceeds with the Sum of Two Single Exponentials

We next sought to determine if accumulation of the slowly relaxing type of depression has any effect on the initial rate at which the RRP refills. We did this by measuring the initial phase of recovery from depletion after the induction of the slowly recovering type of depression, and comparing it to the normal refilling time course of the RRP. The refilling rate itself is accelerated by stimulation and remains elevated for about 20 s (Stevens and Wesseling, 1998). To obtain an accurate measure of the refilling rate for a comparison, we therefore measured the refilling time course starting 20 s after the induction of the slowly recovering depression, and then again 3 min later.

We presented autapses with two pairs of hypertonic solution challenges, the first pair starting 20 s after stimulating the cells with 300 action potentials at 9 Hz, and the second pair starting 3 min after the end of the first pair. The first application of each pair served to empty the pool, and the second provided an estimate for the amount of recovery in the experimentally varied interchallenge interval. The pool content during the second challenge of each pair was compared to the estimated

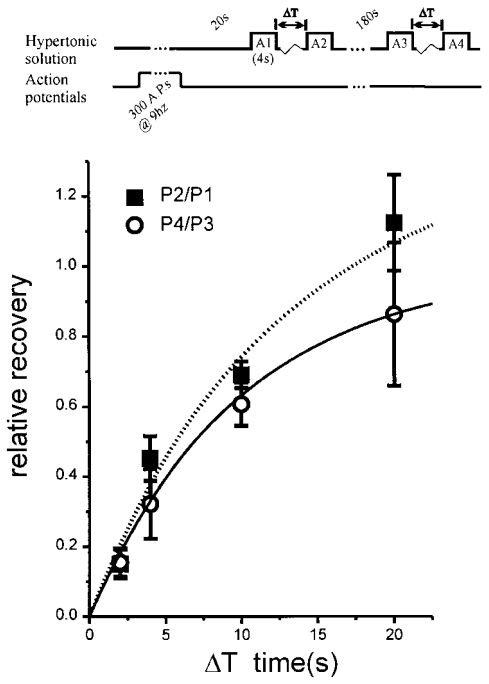

Figure 2. The Initial Component of Recovery Is Unchanged after Depression of the RRP

Depression was induced in autapses by 300 action potentials (9 Hz). The refilling of the RRP was then monitored with two pairs of hypertonic solution challenges—one starting 20 s after the induction of depression and the other 3 min later, as indicated in the diagram at top. The individual hypertonic challenges of each pair were separated in time by an experimentally varied interval (ΔT). The ratio of the estimated pool contents during the second application of each pair (A2 and A4) was compared to the pool contents during the first application (A1 and A3, respectively). Both ratios (squares indicate 20 s after the induction of depression, circles indicate 3 min later, mean ± SEM, at least three observations per data point) are plotted against ΔT. The recovery of the RRP proceeded like a single exponential with a 10 s time constant normally (solid line), and, as predicted, like the weighted sum of that exponential and another one with a time constant of 60 s when the pool had been depressed 20 s earlier by the electrical stimulation (dotted line). The weighting was calculated from the amount of accumulated depression persisting 20 s after the electrical stimulation (P1/P3 = 59%; see text).

pool size determined by the first challenge, and both comparisons are plotted in Figure 2.

Figure 2 shows that accumulation of the slowly recovering component of depression of the RRP is not accompanied by a change in the previously reported refilling rate (Stevens and Tsujimoto, 1995). The high-frequency electrical stimulation induced a substantial amount of the slowly recovering component of depression. Twenty seconds after the long train of action potentials, the size of the RRP was 59% ± 30.4% of the fully recovered size (assuming 5% rundown over 3 min). The refilling time course 3 min after stimulation was well fitted by a single exponential with a time constant of 10 s (Figure 2), a typical value for refilling (Stevens and Tsujimoto, 1995; Stevens and Wesseling, 1998). The relative rate of recovery was slightly faster during the 20 s that started 24 s after the end of the depressive electrical stimulation (Figure 2), but the concurrent, slowly recovering component of depression accounts for this small discrepancy. Both recovery time courses are well fitted by the weighted sum of two exponentials; that is:

$$s(t) = f(1 - e^{-t/\tau_f}) + (1 - f)(1 - e^{-t/\tau_s}) \quad (1)$$

where s(t) is the normalized number of vesicles in the RRP (s = 1 if the pool is completely full, and s = 0 if the pool is totally empty); f is the weighting of the more rapidly recovering exponential (f = 0.59 in the experimental case here, and 3 min later f = 1); τ_s is the time constant of recovery for the slower process estimated from Figure 1 (60 s); and τ_f is the refilling time constant of the RRP (10 s in this case). After the induction of short-term synaptic depression, then, the RRP recovers with the weighted sum of two exponentials, the first originally identified as characteristic of the refilling process, and a slower one with a time constant of about 1 min.

Slowly Recovering Component of Depression Is Presynaptic

We have estimated the recovery of the RRP from the total postsynaptic charge transferred when hypertonic solution is applied to a population of autapses. This method for estimating pool size assumes that the postsynaptic response to individual exocytotic events is unaffected by the electrical stimulation used to produce depression. In the experiments described so far, the individual quantal miniature excitatory postsynaptic currents (mEPSCs) induced by the hypertonic challenges could not be clearly resolved, because isolated neurons typically have many autapses and a total releasable pool that contains hundreds of quanta. In most preparations, then, many vesicles undergo exocytosis nearly simultaneously in response to hypertonic challenges, and thus individual events are obscured. Most long-lasting forms of short-term depression have a presynaptic origin (reviewed by Zucker, 1989), but in some types of synapses at least one component of depression has been shown to be due to postsynaptic changes in the function of neurotransmitter receptors (Trussell et al., 1993; Otis et al., 1996). Stevens and Tsujimoto (1995) report that the hypertonic application itself does not affect the size of the mEPSCs, and Stevens and Wesseling (1998) demonstrate that short trains of action potentials do not change the quantal size in the hippocampal autapse preparation either. In order to verify that our estimates of relative pool size by charge transfer are valid for this study, we must know if long trains of action potentials leave mEPSC size unaffected.

To make sure that the observed long-lasting decrease in the RRP size estimated here is not simply caused by a reduction in the size of the mEPSCs recorded during the hypertonic challenge, then, we induced the slowly recovering type of depression in autapses at which the individual mEPSCs could be identified throughout the hypertonic solution application. One thousand action potentials were evoked (9 Hz), and then hypertonic solution was applied twice, once 10 s after the end of electrical stimulation and then again 3 min later. The quantal rate and size were measured during each osmotic challenge. A comparison of the quanta released after the induction of depression with ones measured when the

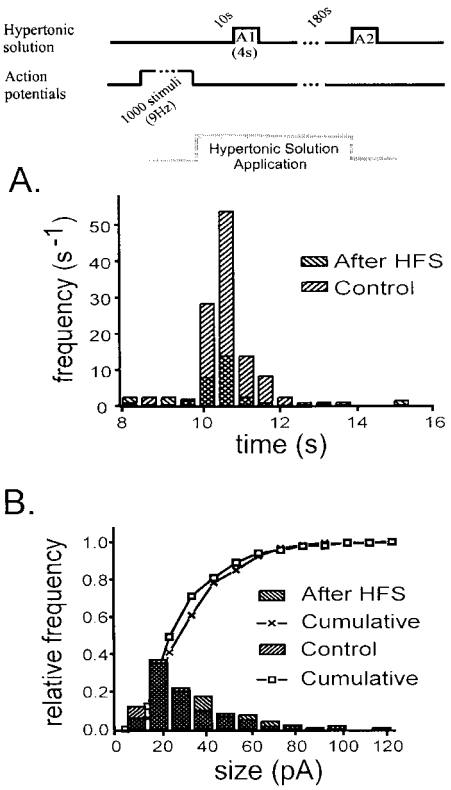

Figure 3. The Slowly Recovering Component of Depression Is Presynaptic

Ten seconds, and again 180 s later, after the induction of slowly recovering depression (1000 action potentials at 9 Hz), hyperosmotic challenges were presented to autapses that responded with easily distinguished mEPSCs.

(A) Average rate of mEPSC occurrence as a function of time during application of hypertonic solution. The evoked mEPSC rate 10 s after high-frequency stimulation (negative slope diagonals) was only about one-fifth the rate after recovery (positive slope diagonals).

(B) Cumulative and noncumulative histograms of mEPSC size during the same hypertonic challenges (conditions for each histogram are symbolized as indicated in the figure). The sizes of the mEPSCs were not significantly different in the two cases (Kolmogorov–Smirnoff two sample test). Data are combined from four experiments on a single isolated cell.

autapses had recovered show that the electrical stimulation did substantially decrease the mEPSC rate (Figure 3A) recorded during the first hypertonic challenge, and did so by an amount that was similar to the total amount of depression. The stimulation did not, however, reduce the size of the quanta (Figure 3B). We conclude, then, that our experimental manipulations do not affect the size of the mEPSCs but rather induce a slowly recovering decrease in the size of the RRP.

Slowly Recovering Component of Depression Is Not Caused by an Increase in the Spontaneous Rate of Exocytosis

High-frequency bursts of action potentials are known to transiently increase the spontaneous rate of mEPSC release. Those fusion events are probably drawn from the RRP (C. F. S. and J. Williams, unpublished data) and if frequent enough could result in a partial depletion of the pool. A large transient increase in this spontaneous rate that decayed away with a 60 s time constant could, therefore, conceivably cause just the sort of slow recovery from depression of the RRP that we observe here. We can quickly rule out this hypothesis, however. While the asynchronous quantal rate was slightly (<15%) elevated for the first few seconds after 111 s of 9 Hz stimulation, the spontaneous mEPSC frequency was actually below normal by 20 s after the last action potential—presumably reflecting the reduced size of the RRP—and then slowly recovered to the baseline frequency over the next several minutes.

Accumulation of the Slow Component Depends upon Exocytosis

Synaptic depression has long been thought to depend on the amount of prior release, in that it has been postulated to result from the exhaustion of pools of vesicles within the presynaptic terminal (see Zucker, 1989). But the long trains of action potentials that are required to induce a substantial amount of the slowly recovering phase of depression reported here have other incidental effects on the presynaptic terminal. For example, high-frequency stimulation is known to cause an intracellular accumulation of sodium ions, which has also been hypothesized to trigger synaptic depression (reviewed by Zucker, 1989).

We sought to determine if the repetitive electrical activity of action potentials alone was enough to induce the slowly recovering phase of depression. By lowering the extracellular calcium to 0.25 mM, we were able to prevent much of the action potential–coupled vesicular exocytosis during high-frequency stimulation (Dodge and Rahamimoff, 1967). The synapses stimulated under such conditions did not accumulate any of the slowly recovering phase of depression of the RRP (Figure 4). We therefore conclude that the accumulation of this type of depression is some downstream result of the calcium influx, possibly the exocytosis that accompanies action potentials.

We next sought to determine if the slow phase of recovery from depression could be manipulated by exocytosis triggered in a way that is not associated with a coincidental rise in intracellular calcium. Stevens and coworkers (Rosenmund and Stevens, 1996; Stevens and Wesseling, 1998) have shown that hypertonic solutions cause exocytosis in just such a calcium-independent way. We reasoned that if the slowly recovering phase of depression were a function of the cumulative amount of exocytosis, then release induced by a hypertonic challenge should be just as effective at causing it to accumulate as release triggered by long trains of electrical stimulation. Little if any of the slowly recovering phase of depression can be induced by a single 4 s application of hypertonic solution (Stevens and Tsujimoto, 1995). But such a challenge only releases a single RRP full of vesicles, and long trains of action potentials release much more than that because the pool refills continually during extended stimulation (see Stevens and Wesseling, 1998). Attempting to match the amount of exocytosis induced by long electrical stimulation protocols with repeated or long applications of hypertonic solution turns out to be impractical. We therefore tested

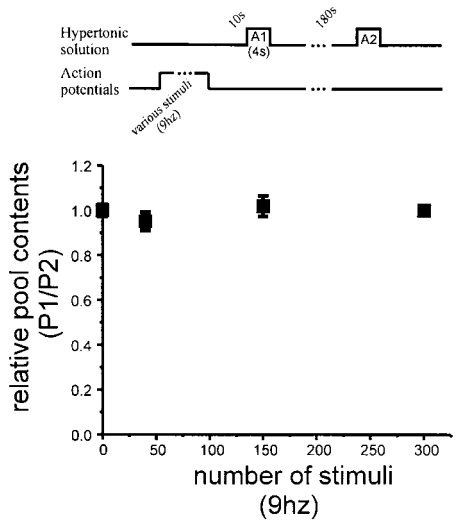

Figure 4. The Slowly Recovering Component of Depression Is Not Induced under Low Calcium Conditions

Calcium was reduced to 0.25 mM in the extracellular solution, and the size of the RRP was monitored 10 s (as indicated in the illustration at top) after the induction of an experimentally varied number of action potentials (9 Hz). Plotted is the amount of depression calculated 10 s after stimulations containing as many as 300 action potentials versus the amount of depression. Data are corrected for rundown as noted in the text (mean ± SEM, at least four observations per data point).

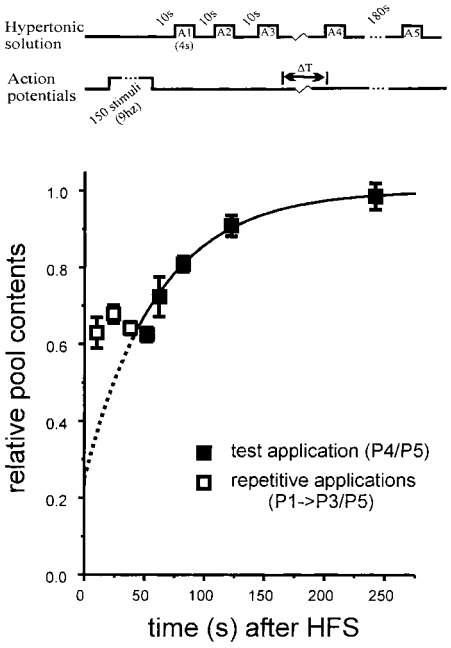

Figure 5. Repeated Applications of Hypertonic Solution Retard the Recovery of the Slowly Recovering Component of Depression of the RRP

Depression was induced in autapses with 9 Hz stimulation (150 action potentials). As indicated in the illustration at the top, the autapses were then probed with hypertonic solution once every 10 s, three times in succession (A1, A2, and A3), and then the recovery was monitored as for Figure 1 (applications A4 and A5). Data are plotted as the fractional pool contents versus time after electrical stimulation (mean ± SEM, three cells, 3–5 trials [closed squares] and 15 trials [open squares]). The first three repetitive applications (open squares) blocked the recovery from depression, which then proceeded as usual (closed squares). The theoretical curve is a single exponential with a 60 s time constant.

to see if release induced by hypertonic challenges could lengthen the slow time course of recovery of the RRP after the previous induction of depression by action potentials.

Figure 5 shows that the slow phase of recovery from depression can be prolonged by the repetitive application of hypertonic solution. Depression was induced in autapses with a train of 150 action potentials evoked at 9 Hz. Hypertonic solution was then applied five times. The first three applications were presented at 10 s intervals after the end of the electrical stimulation. These osmotic challenges prevented the RRP from recovering from the slow component of depression. After the third application, the recovery of the pool was monitored with two more hypertonic challenges in a way that was similar to the experiments for Figure 1. The delay between the third and fourth challenge was varied experimentally, and the fifth hypertonic challenge was presented 3 min after the end of the fourth one when the RRP had recovered completely. The amount of recovery was calculated from the estimated pool size at each of the hypertonic solution presentations divided by the pool size at the fifth one. The data were corrected for an estimated 5% rundown over 3 min. Figure 5 demonstrates that the repetitive challenges with hypertonic solution can prolong the slow phase of recovery from depression of the RRP. After the repetitive challenges, the pool recovered with a time course similar to the one in Figure 1.

Accumulation of the Slow Component Depends upon the History of Synaptic Use

Figure 6 shows the relationship between the amount of exocytosis induced by the high-frequency stimulation and the resulting accumulation of the slowly recovering component of depression. The experiments presented here are similar to the ones detailed for Figure 1, except this time we varied the duration of the electrical stimulation instead of the time between the train of action potentials and the first hypertonic challenge. For each experiment, we applied hypertonic solution to autapses twice. The first challenge was presented 20 s after the end of the electrical stimulation, when we could be sure that the faster process had mostly run its course. The second challenge was presented 3 min after the first one and provided a standardizing measure of the recovered pool size.

Since induction of the slow component of recovery is triggered by exocytosis, we plotted the data in Figure 6 against the amount of exocytosis produced during the electrical stimulation. Because the synapses used in this study depress quickly during high-frequency stimulation, the amount of exocytosis is not proportional to the length of the train of action potentials. We, therefore, used the sum of the sizes of the synaptic responses to individual action potentials as our measure of cumulative exocytosis. The postsynaptic current responses in the autapse preparation overlap with the presynaptic regenerative currents associated with the action potential, and so, to mitigate the impact of this contamination,

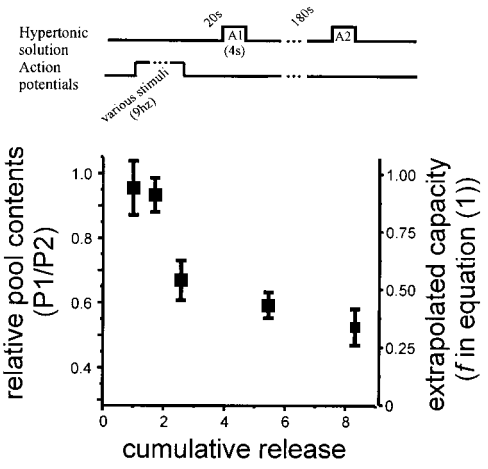

Figure 6. The Slowly Recovering Component of Depression Accumulates with the Amount of Exocytosis Produced during Extended Trains of Action Potentials

Depression was induced in autapses with high-frequency electrical stimulations (9 Hz) of various lengths. The recovery of the RRP was then monitored 20 s later with a single hypertonic solution presentation (A1), and then with another 3 min later (A2), as diagramed at top. The amount of recovery was calculated as the ratio of the estimated pool size at A1 (P1) divided by the size at A2 (P2) and plotted against the estimated relative cumulative release during stimulation trains consisting of 36, 72, 150, 540, and 1000 action potentials (mean ± SEM, at least three observation per data point). Cumulative release was estimated as the summed responses to all of the action potentials and normalized by the responses to the first 36 (see text). Data are corrected for continual rundown as noted in the text. The ordinate on the right indicates the relative weight on the quickly recovering component immediately after the electrical stimulation, assuming a single exponential recovery with a time constant of 60 s.

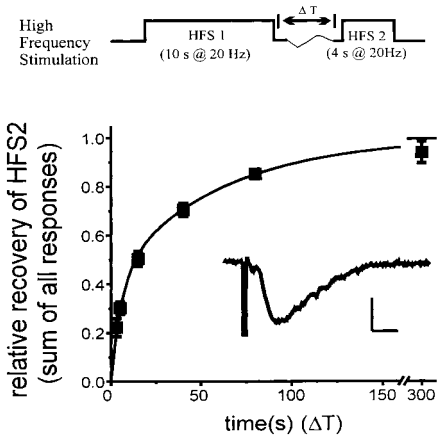

Figure 7. Synapses in Hippocampal Slices Exhibit Both Components of Depression

Depression was induced by a 10 s stimulus delivered at 20 Hz to Schaffer collaterals in mouse hippocampal slices, and then recovery from depression was monitored with another high-frequency stimulation started after a delay (ΔT) as diagramed at the top of the figure. The recovery of the RRP was followed by summing the amplitudes of responses evoked during a 4 s stimulus train (20 Hz). The relative recovery (average ± SEM for three slices with at least five trials per point) is plotted as a function of ΔT (s). The inset is a typical record with the scale bars indicating 0.5 mV and 5 ms. The smooth curve is Equation 1 with time constants of 6 and 60 s and with f = 0.43.

we took the current integral beginning 10 ms after the action potential as our basic measure of synaptic size. Figure 6 shows that the slow component of depression accumulates progressively with longer synaptic use.

Recovery of the RRP Has the Same Properties in Hippocampal Slices

The experiments described above were conducted on hippocampal cells grown in cell culture, and the recovery of the RRP was followed by measuring the exocytosis induced by osmotic shocks. Could the slow recovery characterized here somehow be an artifact of either our cell culture system or of the method employed to evoke release of the RRP? To investigate these questions, we have repeated our main observation (Figure 1) in hippocampal slices with a method that does not involve the use of hypertonic solution.

As noted by Stevens and Wesseling (1999), the usual way of monitoring recovery from depression—determining the size of the response to single action potentials at various times after the induction of depression—has the problem that the high frequency stimulation needed to induce the phenomenon also triggers elements of synaptic enhancement that mask the true extent of depression. The responses to single action potentials thus give an erroneous impression of the time course over which the RRP recovers. We have circumvented this problem in slice experiments by using 4 s trains of action potentials (20 Hz) instead of osmotic challenges to estimate the pool size and thereby monitor its recovery.

Figure 7 shows that the RRPs of synapses in hippocampal slices recover with the same double exponential time course described above for the cell culture system. Schaffer collaterals were stimulated repetitively in transverse mouse hippocampal slices with a silver/silver chloride stimulating electrode. The responses were measured by recording field potentials generated by CA1 pyramidal cells with a recording electrode placed about two-thirds of the way up the apical dendritic trees. To monitor baseline changes, responses were first recorded at low frequency (once every 8 s). Once a stable baseline had been maintained for at least 8 min, two high-frequency trains of action potentials were evoked at 20 Hz for each experiment. The first train was used to induce short-term depression and lasted 10 s (200 stimuli). The second stimulation was started at an experimentally varied amount of time after the end of the first one. After the second high-frequency train, the preparation was again stimulated slowly for several minutes to ensure complete recovery. The slice preparations lasted several (5–10) hours, allowing us to conduct many experiments on the same pathway.

We use the sum of the first 80 postsynaptic responses produced by the second train as our basic measure of recovery over time. The stimulation protocols used here do not depress the synaptic response completely, presumably because the emptied RRP is continuously restocked with fresh vesicles. To correct for this offset, and to guard against possible long-term changes to our preparation, measurements after the various recovery intervals were interleaved with measurements made

when no time was allowed for recovery between the two high-frequency stimulations. The sum of these 80 responses was then subtracted from our basic measure, which was then normalized by the corresponding corrected sum of the first 80 responses of the first stimulation. The normalized amount of recovery is plotted against the experimental rest interval in Figure 7.

As with the culture experiments described earlier, depression in the slice recovered as the sum of two exponentials with time constants of about 6 and 60 s (see Figure 7). Thus, the phenomenon analyzed here occurs in slices as well as in culture, and its properties appear not to depend on the use of hypertonic solution to estimate pool size.

Discussion

Here, we characterize a slowly recovering form of synaptic depression. The time course of recovery is well described by a single exponential with a fixed time constant of about 1 min, and the magnitude of this component of depression is a function of the quantity of transmitter released during stimulation. Previous work has identified a more quickly recovering component of depression of the RRP, which was termed depletion (Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996; Stevens and Wesseling, 1998). Depletion also recovers with an approximately exponential time course, and so the total recovery of the RRP can be described by the weighted sum of two exponentials—one with a time constant typically under 10 s (Stevens and Wesseling, 1998), and the other with a time constant of about 1 min—as described by Equation 1.

What Is the Mechanism?

The goal of this section is to examine several models that can account for the data described earlier; that is, we explore the possible mechanisms underlying Equation 1. The classical theories ascribe the slow recovery from the depression that follows prolonged synaptic use to a depletion of the reserve pool of vesicles (Birks and MacIntosh, 1961; Elmqvist and Quastel, 1965; Magleby, 1987; Zucker, 1989; Liu and Tsien, 1995b), but we shall conclude below that this type of model is inconsistent with our observations and other data. One possible alternative involves a change in the capacity of the RRP, perhaps due to the transient masking of vesicle docking sites at the active zone, that might occur with heavy synapse use. These models are considered in turn.

In the discussion that follows, we need to distinguish between the capacity of a pool and its size; the capacity is the total number of vesicles a pool can hold, and the size is the number it actually contains at a particular time. When a pool is full, its size equals its capacity. If a pool were half full, its size would be half of its capacity. The capacities of the RRP and the reserve pool are unchanging if they are measured repeatedly at sufficiently long intervals (Stevens and Wesseling, 1998; Murthy and Stevens, 1999).

The important quantitative points embodied in Equation 1 are that both the short and long time constants are independent of how much the synapses have been used, and that the relative sizes of the fast and slow components depend upon the amount of prior use.

Classical Depletion Model

The original idea of vesicular mobilization had the readily releasable and reserve pools of transmitter in a steady-state equilibrium with each other, so that depletion of the reserve pool was reflected in the size of the RRP. This model will give Equation 1 as shown in the Appendix, but that formulation is inconsistent with the recent data of Murthy and Stevens (1999). According to the classical model, the steady-state size of the RRP relative to the reserve pool is determined by the rates at which vesicles move between the two pools. Murthy and Stevens (1999) find that the reserve pool is about twice the size of the readily releasable one, which would mean that vesicles would have to enter the RRP at a rate that is one-third the sum of the entry and exit rates (see the Appendix). The rate of transfer out of the RRP would therefore have to proceed more quickly than the rate at which vesicles dock and prime for release. But Murthy and Stevens (1999) find that the exit rate is actually much slower (about once per 2 min per vesicle) than the rate at which the RRP refills, an observation that makes the classical model untenable.

The spirit of the classical model is that the rate of RRP refilling depends upon the number of reserve vesicles (i.e., mass action). That is, after extensive exocytosis, the RRP recovers more slowly because the reserve pool is depleted. The essence of the classical view can still be captured in an extension of the original idea that avoids the problem outlined above if the RRP is assigned a maximum capacity (which it did not have in the classical model). But mass action models such as these cannot account for the additional observation presented here that the fast component of recovery always proceeds with the same time constant no matter the size of the reserve pool. The extended model can give Equation 1 for the particular special case where (1) the rates of transfer into and out of the RRP are approximately equal and (2) the reserve pool is much larger than the readily releasable one (see the Appendix). But, since both of these conditions are contradicted by the Murthy and Stevens (1999) data, we conclude that, if their findings are correct, the classical explanation for the slowly recovering depression is unsatisfactory.

Variable Capacity of the RRP

An alternative model that accounts for all of the data has the capacity of the RRP decreasing with extended exocytosis and returning to the resting capacity with the time constant τ_s. The fast time constant τ_f would represent the refilling of the RRP as usual. This model gives Equation 1 as described in the Appendix and does not place restrictions on the rates of exchange between the RRP and the reserve store.

This alternative model has some compelling biological features. The average hippocampal synapse has an active zone—the region of presynaptic membrane where vesicles dock—of about 0.04 μm^2 (Schikorski and Stevens, 1997). Docking to this structure probably is necessary before vesicles can become readily releasable, since the number of docked vesicles is similar to the number of quanta in the RRP (Schikorski and Stevens, 1997), and vesicles must dock before they can undergo exocytosis. The area of membrane contained in the 90

vesicles released in the Liu and Tsien (1995a, 1995b) experiments would be about 0.15 μm^2, or three to four times the area of the active zone. Because each vesicle contains a complement of membrane proteins that may remain together as a unit during the period after exocytosis and before subsequent endocytosis (Wittich et al., 1994), one can imagine that the unreclaimed vesicles might disrupt the active zone structure and transiently diminish the capacity of the RRP by obscuring docking sites. Morphological evidence for such a disruption of the active zone by heavy use has been reported by Shupliakov et al. (1997). During periods of rest, the pool's capacity would then return to its resting value as the spent vesicular membrane was taken back into the terminal. Twenty seconds after heavy synaptic use, then, a completely full pool—i.e., with all the available docking sites occupied—would contain fewer vesicles than the same pool after a much longer rest.

The preceding presents a specific picture of why the number of available docking sites might decrease with heavy synaptic use, but it is not the only possible version of this class of explanations. One could also imagine that the number of docking sites could be decreased by lack of availability of biochemical components needed for the docking and priming reactions, or that sites are blocked by newly recycled vesicles that have yet to mature.

Relation to Previous Observations

Two recent studies have reported what is probably the same effect we describe here. Von Gersdorff and Matthews (1997) find that a releasable pool of transmitter at a retinal ribbon synapse refills with a time constant of about 8 s. They also report that the "refilling" is slowed after strong synaptic use, although they could not accurately measure the longer refilling time constant (they report a value greater than 20 s). As they point out, the slower refilling that they observe could easily be served by the same mechanism underlying the observations of Liu and Tsien (1995a, 1995b).

The study most comparable to the present one is that by Liu and Tsien (1995a, 1995b), who report a 39 s refilling time constant after the release of about 90 quanta from single synapses by local application of hyperkalemic solution. They estimated their characteristic time constant by fitting their data with a single exponential that is constrained to pass through zero at zero time. Since their first recovery observation was made 30 s after the induction of depression, Liu and Tsien would not have observed the initial rapid refilling that we see. When their data are refitted with a single exponential that is not constrained to pass through the origin, however, but with the recovery time constant set at our value of 60 s, then the initial pool size would be 0.32 of its resting value immediately after depression, a value comparable with our data (Figure 6). Thus, the data of both of these earlier studies are consistent with the same slowly relaxing depression in the capacity of the RRP that we report here.

Experimental Procedures

For the cell culture experiments, the recovery of the RRP was estimated with methods similar to those used by Stevens and Tsujimoto (1995) and Rosenmund and Stevens (1996). Isolated neurons were grown in cell culture as described previously (Furshpan et al., 1976, 1986; Segal and Furshpan, 1990; Bekkers and Stevens, 1991). Round coverslips (12 mm) were coated with 0.15% agarose (type IIa) and allowed to dry in 24-well cell culture plates. A glass chromatography atomizer (Fisher) was then used to spray a particulate mist of substrate solution containing rat tail collagen (0.25 mg/ml; either bought commercially or prepared directly from rat tails as described by Banker and Goslin, 1991), and poly-D-lysine (0.1 mg/ml; CBI). Tissue from the CA1–CA3 regions of newborn mice was dissociated as previously described (Bekkers and Stevens, 1989) and 0.5 ml of a cell suspension diluted to around 3×10^4 cells/ml were added to each well. Neurons were grown in high glucose (20 mM) media containing 10% horse serum for 8–14 days before use.

Isolated neurons were patch clamped in perforated whole-cell mode. The extracellular saline solution usually contained (in mM) 132 NaCl, 2 KCl, 10 glucose, 15 Sorbitol, 10 HEPES, 0.5 $MgCl_2$, and 5 $CaCl_2$. A few experiments were conducted with 2 mM $MgCl_2$ and 0.25 mM $CaCl_2$ instead. 50 μM D(−)APV was added to block NMDA-type glutamate currents. The patch clamp electrode was filled with a solution containing (in mM) 140 K-gluconate, 9 NaCl, 0.2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 1 EGTA, 2 MgATP, and 0.2 LiGTP. Amphotericin (0.1 mg/ml, B-solubilized; Sigma) was added to permeabilize the patch membrane to ions. All salines were adjusted to have a pH around 7.2, and had an osmolarity between 295 and 305 mOsm/kg. Uncontrolled action potentials were evoked presynaptically by transiently depolarizing the cell bodies as described earlier (Bekkers and Stevens, 1991). Hypertonic solution (normal extracellular saline augmented with 500 mM sucrose) was applied by picospritzing from a glass pipette with a tip diameter of between 2 and 3 μm. A vacuum pipette with a diameter of 10–50 μm was used to clear the hypertonic solution rapidly from the preparation. To accurately estimate the fractional fullness of the RRP, we corrected the integral of current flow caused by hypertonic solution by subtracting away the amount of steady-state refilling and exocytosis that occurred during hypertonic challenges (Stevens and Wesseling, 1998).

Slice experiments were performed on transverse slices prepared from the hippocampi of 3- to 5-week-old mice. Mice were anesthetized with an intraperitoneal injection of chloral hydrate and decapitated soon after the disappearance of reflexive reactions to tail and foot pinches. The brain was rapidly removed and bathed in a chilled solution that had most of the sodium ions replaced with sucrose (in mM: 230 sucrose, 10.25 NaH_2PO_4, 26 $NaHCO_3$, 30.5 KCl, 10.3 $MgCl_2$, and 20.6 $CaCl_2$ bubbled with 95% O_2, 5% CO_2). The cerebellum and brain stem were dissected away and 400 μm thick coronal slices of the remaining brain were cut using a vibrating microtome. Hippocampal segments were dissected free, and area CA3 was removed. The hippocampal slices were then gently washed three or four times in the recording solution (in mM: 120 NaCl, 10.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, 30.5 KCl, 20.5 $CaCl_2$, 10.3 $MgCl_2$, 5 picrotoxin, and 50 μM D(−)APV), which was bubbled with 95% O_2, 5% CO_2 for at least 20 min prior to use. The slices were maintained in an interface slice chamber humidified with 95% O_2, 5% CO_2 at room temperature for between 2 and 10 hr before being transferred to the submerged recording chamber.

Slices were submerged in the recording chamber with a nylon mesh affixed to a platinum anchor. The solution in the ~1 ml recording chamber was exchanged at a rate of about 1 ml/min with continuously bubbled recording solution. A monopolar silver/silver chloride stimulating electrode inserted into a glass pipette (tip diameter between 20 and 40 μm) filled with recording solution was placed in the Schaffer collaterals. Excitatory postsynaptic potentials (EPSPs) were evoked by using up to 20 V stimuli of 0.1 ms duration. All experiments reported here were conducted at room temperature (23°C–25°C). Extracellular recordings were made with glass pipettes (2 μm tip diameters), filled with recording solution, and placed in the distal one-third of the stratum radiatum between 300 and 800 μm away from the stimulator toward the subicular end of the hippocampus. Recordings were only accepted if the peak synaptic potential had at least twice the amplitude of the fiber volley, and there was no discernible population spike when the synapses were stimulated once every 8 s. Response size was measured as the initial slope of the rising phase of the postsynaptic response, estimated by fitting the rising segment between 30% and 60% of the peak with a linear

least-squares fit. Stimulation rates reported in the text are accurate to within ±10%.

Appendix

The Classical Model

According to the classical model for depletion (Birks and MacIntosh, 1961; Elmqvist and Quastel, 1965; Magleby, 1987; Zucker, 1989; Liu and Tsien, 1995b; Wu and Betz, 1998), two collections of synaptic vesicles are present in resting boutons: the readily releasable vesicles (the D pool here, for docked and primed) and the reserve pool (the R pool). The R pool is refilled with a rate constant k by recycling of spent vesicles (the U pool of used vesicles), vesicles in R dock and prime (move from R to D) with a rate constant a, and those in D return to the reserve pool (vesicles in D returning to R) with a rate constant b. The classical model is, in the absence of vesicular release, thus represented by the kinetic scheme:

$$U \xrightarrow{k} R \underset{b}{\overset{a}{\rightleftarrows}} D$$

We denote the number of vesicles in the R pool as N(t) and the number in the D pool as n(t); note that pool sizes are given as a fraction of the total number of recycling vesicles, so that if half of all the vesicles were in the reserve pool, we would have N = 0.5. We assume that the total recycling pool is of constant size so that the fraction of vesicles in the U pool is 1 − n(t) − N(t). The equation that describes the rate at which the size of the D pool changes is

$$\frac{dn(t)}{dt} = aN(t) - bn(t) \tag{1A}$$

We stress that this equation is valid only when no release occurs after t = 0, although the pool contents at this time of course depend on exocytosis before t = 0. The corresponding gain–loss equation for the rate of change of the number of vesicles in the R pool is:

$$\frac{dN(t)}{dt} = -aN(t) + bn(t) + k[1 - N(t) - n(t)] \tag{2A}$$

The quantity that we measure in this study is proportional to n(t), and a second-order differential equation for n(t) can be found by eliminating N(t) between Equations 1A and 2A. This equation has the solution, found with standard methods,

$$n(t) = a\frac{a + b - k - (N(0)(a + b) - k)e^{-t/\tau_f} - (1 - N(0))(a + b)e^{-t/\tau_s}}{(a + b)(a + b - k)} \tag{3A}$$

with

$$\tau_f = \frac{1}{(a + b)} \text{ and } \tau_s = \frac{1}{k}.$$

Here, we have assumed that the D pool starts completely empty at t = 0 and have denoted the initial size of the R pool by N(0).

If we define s(t) by

$$s(t) = n(t)\frac{(a + b)}{a}$$

and f and (1 − f) by

$$f = \frac{N(0)(a + b) - k}{a + b - k}$$

and

$$1 - f = \frac{(a + b)(1 - N(0))}{a + b - k}$$

Equation 1 results. According to Equation 3A, the resting size of the D pool is a/(a + b) and that of the R pool is b/(a + b), because N + n = 1 when no vesicles are waiting to be recycled (as occurs in the resting state). Murthy and Stevens (1999) find that the D pool is about one-third the size of the total recycling pool, so a/(a + b) = 1/3 or b = 2a if the classical model is correct. But Murthy and Stevens also find that b = a/5, a ~10-fold difference from the prediction made by the classical model. The data are thus inconsistent with the classical explanation for the slow component of depression.

The Extended Classical Model

The classical model is inaccurate in part because the sizes of the hypothetical pools are allowed to vary without limit. This means that the size of the D pool, for example, is set entirely by the rate constants a and b. Can this model be rescued by placing an upper limit on the number of docking sites so that D pool size is no longer determined solely by the rates a and b? The essence of the classical idea is that the slow component of depression occurs when the reserve pool is depleted because the RRP refills at a rate that is proportional to the number of reserve vesicles. This concept can be incorporated into a more complex model where the pools have restricted capacities. We show below, however, that this extended model is also inconsistent with our observations.

We extend the classical model by denoting the capacity of the R pool by N_0 and that of the D pool by n_0. In this model, the rate of transfer from one pool to the other is jointly proportional to the number of vesicles in the source pool and to the number of vacancies in the destination pool. For example, the rate of transfer from the R pool to the D pool would be $aN(t)[n_0 - n(t)]$, where $[n_0 - n(t)]$ is the number of D pool vacancies, N(t) is the number of vesicles in the R pool (the source), n(t) is the number in the D pool (the destination), and a is the rate constant for the R to D transfer as in the classical model described above.

For the D pool with no release occurring, the rate of change of the contents dn(t)/dt is equal to the rate at which vesicles are supplied from the R pool (the first term on the right of Equation 4A) minus the loss back to the R pool from the D pool (the second term on the right):

$$\begin{aligned}\frac{dn(t)}{dt} &= a[n_0 - n(t)]N(t) - b[N_0 - N(t)]n(t) \\ &= -[(a - b)N(t) + bN_0]n(t) + an_0N(t)\end{aligned} \tag{4A}$$

where b is the rate constant for the D to R transfer. For dN(t)/dt, the rate of change of the number of vesicles in the R pool, the corresponding gain–loss equation is:

$$\begin{aligned}\frac{dN(t)}{dt} &= -a[n_0 - n(t)]N(t) + b[N_0 - N(t)]n(t) + k[N_0 - N(t)] \\ &= -(an_0 + k)N(t) + bN_0n(t) + kN_0 + (a - b)N(t)n(t)\end{aligned} \tag{5A}$$

where k is the rate of R pool replenishment as in the classical model. The first term on the right is the rate of vesicular loss from the R pool to the D pool, the second is the rate of the R pool gain from the D pool, and the last term is the rate at which the depleted R pool regenerates.

A second-order differential equation for n(t) can be found by eliminating N(t) between Equations 4A and 5A. The full treatment of this equation would require lengthy discussion but fortunately is not required for our present purposes.

According to the classical depletion concept, the rapid recovery of the D pool is due to a fast equilibration between the R and D pools, whereas the slow process proceeds as the R pool is replenished. If the capacity of the R pool (N_0) is small enough so that the pool changes in size as it loses vesicles to the D pool, as suggested by Murthy and colleagues (Murthy et al., 1997; Murthy and Stevens, 1999), the portion of the D pool ascribed to the slow component exponential (1 − f in Equation 1) would have to be large even after short stimulations that only empty the RRP once. This is not the case. The slow component of recovery only begins to play a substantial role after the release of several RRPs full of vesicles (see Figure 6; Geppert et al., 1994; Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996; Stevens and Wesseling, 1998).

On the other hand, even if the capacity of the R pool (N_0) were large compared to that of the D pool (n_0), the extended classical model still could not account for our data because the rate of transfer out of the D pool and back to the R is far too slow. In the case where the R pool does not lose a substantial amount of material as the D pool refills, we can consider N(t) to be approximately constant over the time course of rapid R pool refilling (~10 s) because the R pool regenerates slowly (>60 s). N(t) can thus be approximated

by N(0), and Equation 4A can be easily solved by standard methods to yield:

$$n(t) \approx \frac{an_0N(0)}{(a - b)N(0) + bN_0}(1 - e^{-t/\tau_f}) \quad (6A)$$

with

$$\tau_f \approx \frac{1}{(a - b)N(0) + bN_0}$$

Unless a and b are approximately equal, the rapid time constant (τ_f) in Equation 6A depends strongly upon the initial size of the R pool (N(0)). But, our observation here is that τ_f is independent of the initial fullness of the R pool over a range of $N(0) = N_0$ to $N(0) = 0.3N_0$ (Figure 2). Thus, the extended classical model can only be consistent with our data when a and b take on similar values, so that the term $(a - b)N(0)$ in the denominator of the expression for τ_f would vanish. As we noted above, however, the rate of transfer out of the RRP (b) is much slower than the rate of docking and priming (Murthy and Stevens, 1999).

In summary, the extended classical model requires that the reserve pool be much larger than the readily releasable one, and that the forward and reverse rates of transfer between the pools be comparable. Neither of these assumptions is true. The rate of transfer into the RRP from the reserve one is much faster than the reverse reaction (Murthy and Stevens, 1999), and the reserve pool is only about twice the size of the readily releasable one (Murthy et al., 1997; Murthy and Stevens, 1999). We conclude, then, that the extended classical model is not consistent with our data and can be rejected.

A Variable Capacity Model

The picture for this alternative model is that the membrane from fused vesicles disrupts the normal exocytotic machinery by somehow occluding vesicle docking sites in the active zone. Since vesicles must dock to the active zone before priming for release (Llinás et al., 1981), the obstruction would result in a reduction in the capacity of the RRP. The capacity would then recover to its resting value with the clearance of spent membrane via endocytosis and repair of the active zone. The rate of change in the capacity (C) of the RRP then would be described by the gain–loss equation:

$$\frac{dC}{dt} = k(C_{max} - C(t)) - B(t) \quad (7A)$$

where C_{max} is the maximum capacity of the RRP, and

$$k = \frac{1}{60}\,s^{-1}$$

($1/\tau_s$ in Equation 1). B(t) is a function that describes the rate at which the active zone docking sites are occluded by ongoing exocytosis. This function would depend on the history of synaptic use, but the measurements presented here only follow the recovery of the RRP when the rate of exocytosis is negligible. We thus assume that $B(t) = 0$ during the recovery interval described by Equation 1. For the conditions under which Equation 1 holds, then:

$$\frac{dC}{dt} = \alpha(C_{max} - C(t)) \quad (8A)$$

and therefore,

$$C(t) = C(0)e^{-t/\tau_s} + C_{max}(1 - e^{-t/\tau_s}) \quad (9A)$$

Recovery thus proceeds exponentially with the time constant of active zone repair.

Our working picture of refilling for the RRP is that it occurs at a rate that is proportional to the number of vacancies with a rate constant $1/\tau_f$ (Stevens and Tsujimoto, 1995), so during periods of rest the number of vesicles in the RRP n(t) is governed by the equation

$$\frac{dn}{dt} = \frac{1}{\tau_f}(C(t) - n(t)) \quad (10A)$$

which (for the C(t) given in Equation 9A) has the solution

$$n(t) = C_{max} - \frac{\tau_s C_{max} - \tau_s C(0)}{\tau_s - \tau_f}e^{-t/\tau_s} - \frac{\tau_s C(0) - \tau_f C_{max}}{\tau_s - \tau_f}e^{-t/\tau_f} \quad (11A)$$

Since Equation 1 describes the fractional recovery of the RRP, n(t) must be similarly normalized for direct comparison to s(t) in Equation 1. We thus let $s(t) = n(t)/C_{max}$. If we define f to be

$$f = \frac{\tau_s\frac{C(0)}{C_{max}} - \tau_f}{\tau_s - \tau_f},$$

Equation 11A reduces to Equation 1 in the text. In this way, a simple model with a docked vesicle pool capacity that varies with the amount of exocytosis can account for our data.

Acknowledgments

We thank Yukiko Goda and Samuel S.-H. Wang for helpful advice. This work was supported by the Howard Hughes Medical Institute, by the National Institutes of Health grant NS 12961 (C. F. S.), and by a predoctoral fellowship from the National Science Foundation (J. F. W.).

Received July 6, 1999; revised October 5, 1999.

References

Abbott, L.F., Varela, J.A., Sen, K., and Nelson, S.B. (1997). Synaptic depression and cortical gain control. Science *275*, 220–224.

Banker, G., and Goslin, K. (1991). Culturing Nerve Cells (Cambridge, MA: MIT Press).

Bekkers, J.M., and Stevens, C.F. (1989). NMDA and non-NMDA receptors are co-localized at individual excitatory synapses in cultured rat hippocampus. Nature *341*, 230–233.

Bekkers, J.M., and Stevens, C.F. (1990). Presynaptic mechanism for long-term potentiation in the hippocampus. Nature *346*, 724–729.

Bekkers, J.M., and Stevens, C.F. (1991). Excitatory and inhibitory autaptic currents in isolated hippocampal neurons maintained in cell culture. Proc. Natl. Acad. Sci. USA *88*, 7834–7838.

Bekkers, J.M., Richerson, G.B., and Stevens, C.F. (1990). Origin of variability in quantal size in cultured hippocampal neurons and hippocampal slice. Proc. Natl. Acad. Sci. USA *87*, 5359–5362.

Bellingham, M.C., and Walmsley, B. (1999). A novel presynaptic inhibitory mechanism underlies paired pulse depression at a fast central synapse. Neuron *23*, 159–170.

Birks, R.I., and MacIntosh, F.C. (1961). Acetylcholine metabolism of a sympathetic ganglion. Can. J. Biochem. Physiol. *39*, 787–827.

Brenowitz, S., David, J., and Trussell, L. (1998). Enhancement of synaptic efficacy by presynaptic GABA(B) receptors. Neuron *20*, 135–141.

Dobrunz, L.E., and Stevens, C.F. (1997). Heterogeneity of release probability, facilitation, and depletion at central synapses. Neuron *18*, 995–1008.

Dodge, F. A., Jr., and Rahamimoff, R. (1967). Co-operative action of calcium ions in transmitter release at the neuromuscular junction. J. Physiol. *193*, 419–432.

Elmqvist, D., and Quastel, D.M.J. (1965). A quantitative study of end-plate potentials in isolated human muscle. J. Physiol. *178*, 505–529.

Furshpan, E.J., MacLeish, P.R., O'Lague, P.H., and Potter, D.D. (1976). Chemical transmission between rat sympathetic neurons and cardiac myocytes developing in microcultures: evidence for cholinergic, adrenergic, and dual-function neurons. Proc. Natl. Acad. Sci. USA *73*, 4225–4229.

Furshpan, E.J., Landis, S.C., Matsumoto, S.G., and Potter, D.D. (1986). Synaptic functions in rat sympathetic neurons in microcultures. I. Secretion of norepinephrine and acetylcholine. J. Neurosci. *6*, 1061–1079.

Geppert, M., Goda, Y., Hammer, R.E., Li, C., Rosahl, T.W., Stevens, C.F., and Südhof, T.C. (1994). Synaptotagmin I: a major Ca^{2+} sensor for transmitter release at a central synapse. Cell *79*, 717–727.

Goda, Y., and Stevens, C.F. (1996). Long-term depression properties in a simple system. Neuron *16*, 103–111.

Goda, Y., and Stevens, C.F. (1998). Readily releasable pool size

changes associated with long term depression. Proc. Natl. Acad. Sci. USA *95*, 1283–1288.

Katz, B. (1969). The Release of Neural Transmitter Substances (Liverpool, UK: Liverpool University Press).

Korn, H., and Faber, D.S. (1991). Quantal analysis and synaptic efficacy in the CNS. Trends Neurosci. *14*, 439–445.

Liley, A.W., and North, K.A.K. (1953). An electrical investigation of effects of repetitive stimulation on mammalian neuromuscular junction. J. Neurophysiol. *16*, 509.

Liu, G., and Tsien, R.W. (1995a). Properties of synaptic transmission at single hippocampal synaptic boutons. Nature *375*, 404–408.

Liu, G., and Tsien, R.W. (1995b). Synaptic transmission at single visualized hippocampal boutons. Neuropharmacology *34*, 1407–1421.

Llinás, R., Steinberg, I.Z., and Walton, K. (1981). Relationship between presynaptic calcium current and postsynaptic potential in squid giant synapse. Biophys. J. *33*, 323–352.

Magleby, K.L. (1987). Short-term changes in synaptic efficacy. In Synaptic Function, G.M. Edelman, V.E. Gall, and K.M. Cowan, eds. (New York: John Wiley and Sons), pp. 21–56.

Murthy, V.N., and Stevens, C.F. (1999). Reversal of synaptic vesicle docking at central synapses. Nat. Neurosci. *2*, 503–507.

Murthy, V.N., Sejnowski, T.J., and Stevens, C.F. (1997). Heterogeneous release properties of visualized individual hippocampal synapses. Neuron *18*, 599–612.

Otis, T., Zhang, S., and Trussell, L.O. (1996). Direct measurement of AMPA receptor desensitization induced by glutamatergic synaptic transmission. J. Neurosci. *16*, 7496–7504.

Rosenmund, C., and Stevens, C.F. (1996). Definition of the readily releasable pool of vesicles at hippocampal synapses. Neuron *16*, 1197–1207.

Ryan, T.A., Ziv, N.E., and Smith, S.J. (1996). Potentiation of evoked vesicle turnover at individually resolved synaptic boutons. Neuron *17*, 125–134.

Schikorski, T., and Stevens, C.F. (1997). Quantitative ultrastructural analysis of hippocampal excitatory synapses. J. Neurosci. *17*, 5858–5867.

Segal, M.M., and Furshpan, E.J. (1990). Epileptiform activity in microcultures containing small numbers of hippocampal neurons. J. Neurophysiol. *64*, 1390–1399.

Shupliakov, O., Low, P., Grabs, D., Gad, H., Chen, H., David, C., Takei, K., De Camilli, P., and Brodin, L. (1997). Synaptic vesicle endocytosis impaired by disruption of dynamin–SH3 domain interactions. Science *276*, 259–263.

Stevens, C.F., and Tsujimoto, T. (1995). Estimates for the pool size of releasable quanta at a single central synapse and for the time required to refill a pool. Proc. Natl. Acad. Sci. USA *92*, 846–849.

Stevens, C.F., and Wang, Y. (1995). Facilitation and depression at single central synapses. Neuron *14*, 795–802.

Stevens, C.F., and Wesseling, J.F. (1998). Activity-dependent modulation of the rate at which synaptic vesicles become available to undergo exocytosis. Neuron *21*, 415–424.

Stevens, C.F., and Wesseling, J.F. (1999). Augmentation is a potentiation of the exocytotic process. Neuron *22*, 139–146.

Takahashi, T., Forsythe, I.D., Tsujimoto, T., Barnes-Davies, M., and Onodera, K. (1996). Presynaptic calcium current modulation by a metabotropic glutamate receptor. Science *274*, 594–597.

Tong, G., and Jahr, C.E. (1994). Multivesicular release from excitatory synapses of cultured hippocampal neurons. Neuron *12*, 51–59.

Trussell, L.O., Zhang, S., and Raman, I.M. (1993). Desensitization of AMPA receptors upon multiquantal neurotransmitter release. Neuron *10*, 1185–1196.

von Gersdorff, H., and Matthews, G. (1997). Depletion and replenishment of vesicle pools at a ribbon-type synaptic terminal. J. Neurosci. *17*, 1919–1927.

Wittich, B., Volknandt, W., and Zimmermann, H. (1994). SV2 and o-rab3 remain associated with recycling synaptic vesicles. J. Neurochem. *63*, 927–937.

Wu, L.G., and Betz, W.J. (1998). Kinetics of synaptic depression and vesicle recycling after tetanic stimulation of frog motor nerve terminals. Biophys. J. *74*, 3003–3009.

Zucker, R.S. (1989). Short-term synaptic plasticity. Annu. Rev. Neurosci. *12*, 13–31.